Nitrogen management in grasslands and forage-based production systems – Role of biological nitrification inhibition (BNI)

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Abstract. Nitrogen (N), being the most critical and essential nutrient for plant growth, largely determines the productivity in both extensive- and intensive- grassland systems. Nitrification and denitrification processes in the soil are the primary drivers generating reactive-N: NO_3^-, N_2O, and NO, and is largely responsible for N-loss and degradation of grasslands. Suppressing nitrification can thus facilitate the retention of soil-N to sustain long-term productivity of grasslands and forage-based production systems. Certain plants can suppress soil nitrification by releasing inhibitors from roots, a phenomenon termed 'biological nitrification inhibition' (BNI). Recent methodological developments (*e.g.* bioluminescence assay to detect BNIs from plant-root systems) led to significant advances in our ability to quantify and characterize BNI function in pasture grasses. Among grass-pastures, BNI-capacity is strongest in low-N adapted grasses such as *Brachiaria humidicola* and weakest in high-N environment grasses such as Italian ryegrass (*Lolium perenne*) and *B. brizantha*. The chemical identity of some of the BNIs produced in plant tissues and released from roots has now been established and their mode of inhibitory action determined on nitrifying bacteria *Nitrosomonas*. Synthesis and release of BNIs is a highly regulated and localized process, triggered by the presence of NH_4^+ in the rhizosphere, which facilitates the release of BNIs close to soil-nitrifier sites. Substantial genotypic variation is found for BNI-capacity in *B. humidicola*, which opens the way for its genetic manipulation. Field studies suggest that *Brachiaria* grasses suppress nitrification and N_2O emissions from soil. The potential for exploiting BNI function (from a genetic improvement and a system perspective) to develop production systems that are low-nitrifying, low N_2O-emitting, economically efficient and ecologically sustainable, will be the subject of discussion.

Keywords: *Brachiaria* spp., grassland productivity, green house gas, nitrogen losses, nitrous oxide emissions, nitrogen-use efficiency.

Introduction

Grasslands are the largest land use, occupying 3.2 billion ha out of 4.9 billion ha of available agricultural land worldwide (Steinfeld *et al*. 2006). In addition, a significant portion of the cultivated land (0.5 billion ha) is used for growing forage grasses and feed-grain crops (*e.g.* sorghum, barley, maize and soybean) to support intensive livestock production (Steinfeld and Wassenaar 2007; Herrero *et al*. 2010, 2011). N-fixation by legumes and mineralization of soil organic matter (SOM) are major N sources in extensive grassland systems.

For intensive grass-pastures, fertilizer-N inputs can reach from 200 to 600 kg N/ha/yr (Galloway *et al*. 2009). Only 30% of the N applied to these intensive pastures is captured in plant-protein and enters into the animal system; the remaining 70% is lost to the environment in reactive-N forms (*i.e.* NO_3^-, N_2O, NO) (Galloway *et al*. 2009).

Nitrogen-use efficiency (NUE) in grassland systems (meat/milk-protein produced/kg plant-protein-N intake) ranges from 5 to 10% depending on milk- or meat-protein as output (van der Hoek 1998). Grazing animals typically retain about 5% of the N they consume as grass and the rest is excreted in urine (about 90% of the total N intake) and dung, which becomes N-source for the pasture (Worthington and Danks 1992). However, much of this N is lost through NO_3^- leaching and gaseous N emissions (N_2O, NO and N_2), causing ecological damage and economic loss (Tilman *et al*. 2002; Steinfield and Wassenaar 2007; Herrero *et al*. 2011; Subbarao *et al*. 2013b).

N loss from agricultural systems impacts the global environment and contributes significantly to global warming

Intensive pasture and feed-grain production systems often have high-nitrifying soil environments (where NO_3^- accounts for >95% of the plant N uptake) which are extremely "leaky" and intrinsically inefficient (Subbarao *et al*. 2012). Nearly 70% of the 150 Tg N-fertilizer globally applied annually to the agricultural systems is lost through NO_3^- leaching or N_2O and NO emissions (Vitousek and Howarath 1991). The annual economic loss is estimated to be about US$90 billion (Subbarao *et al*. 2013b). Fertilizer-N use is projected to reach 300 Tg/y by 2050 (Tilman *et al*. 2001) and N lost from NO_3^- leaching will further intensify (Schlesinger 2009). Currently 17 Tg N is emitted as N_2O and this is expected to quadruple by 2100, due largely to an increase in the use of N-fertilizers (Galloway *et al*. 2008).

Nitrification opens several pathways for N-loss and weakens the soil-N retention capacity in grassland systems

Nitrogen enters grass pasture primarily as N-fertilizer (in intensive systems) or is derived from SOM-mineralization (in extensive systems) or hydrolysis of urea-N from urine excreted from the grazing animals. NH_4^+ is the first inorganic-N product formed as a result of SOM-mineralization-ammonification or urea hydrolysis. Nitrification, the biological oxidation of NH_4^+ to NO_3^-, then opens several pathways for N loss by leaching, and by production of N_2O and NO which are generated by nitrifier-denitrification or heterotrophic-denitrification processes (Davidson and Verchot 2000; Zhu *et al*. 2013). NO_3^- does not readily bind to the soil as it is negatively charged, and it is sufficiently labile to be leached readily below the root zone. Nitrification combined with denitrification is a major driver of global N_2O emissions, the most powerful greenhouse-gas. The global warming potential of N_2O is 300 times greater than that of CO_2 (Hahn and Crutzen 1982).

By contrast, NH_4^+ is held by the negatively charged surfaces of clay minerals and SOM and this reduces the potential for NH_4^+ loss by leaching. Heterotrophic soil microorganisms and pasture roots may also utilize the NH_4^+ converting it to plant proteins or microbial-N, respectively (Fig. 1). Nitrogen flow into the microbial biomass is a temporary form of N immobilization because this N may become available during the growing season of the pasture as a result of turnover in the microbial biomass. Restricting the N-flow to the nitrification pathway by inhibiting soil nitrifier activity facilitates NH_4^+ uptake by plants and this also allows N-flow into microbial pool (Hodge *et al*. 2000). This should help to keep N cycling in the soil and create a slow-release N pool to sustain grassland productivity (Fig. 1). Many plants have the ability to use NH_4^+ or NO_3^- as their N source (Haynes and Goh 1978; Boudsocq *et al*. 2012). Reducing nitrification rates in agricultural systems does not alter the intrinsic ability of plants to absorb N. However, it does increase N retention time in the root zone as NH_4^+ providing additional time for plants to absorb N. Many of the advantages associated with inhibiting nitrification in improving productivity and NUE of intensive grassland systems and feed-grain production systems have been demonstrated using chemical nitrification inhibitors (Subbarao *et al*. 2006a; Dennis *et al*. 2012).

Biological nitrification inhibition (BNI)

The BNI concept

The ability to produce and release nitrification inhibitors from plant roots to suppress soil nitrifier-activity is termed, 'biological nitrification inhibition' (Fig. 1). Nitrification largely determines the N-cycling efficiency (*i.e*. proportion of N that stays in the ecosystem during a complete N-cycling loop); BNI function thus, has the potential to improve agronomic-NUE (Subbarao *et al*. 2012; 2013b). This was also shown by *in situ* measures showing that tropical grasses that inhibit nitrification exhibit a 2-fold greater productivity than those that lack such ability (Lata 1999). Models predicted that ecosysem properties such as biomass, productivity and N losses are indeed linked to grasses ability to control nitrification but also to their preference for ammonium versus nitrate (Boudsocq *et al*. 2012).

BNI characterization in pasture grasses

Recent methodological advances have facilitated the detection and quantification of nitrification inhibitors from intact plant roots using a recombinant *Nitrosomonas* construct (Subbarao *et al*. 2006b). Nitrification inhibitors released from roots measured as 'BNI-activity', are expressed in ATU (allylthiourea unit) (ATU) and this ability is termed BNI-capacity (Subbarao *et al*. 2007b). Tropical pasture grasses showed a wide range in the BNI-capacity of their root systems. *B. humidicola* forage grasses that are adapted to low-N production environments of South American Savannas showed the greatest BNI-capacity (range from 15 to 50 ATU/g root dry wt./d) (Subbarao *et al*. 2007b). By contrast, *Lolium perenne*, *B. brizantha* and *P. maximum*, that are adapted to high-N environments, showed the least BNI-capacity (2 to 5 ATU/g root dry wt./d) (Fig. 2). Sorghum is the only field crop that showed significant BNI-capacity (5 to 10 ATU/g root dry wt./d) among the cereal and legume crops evaluated (Subbarao *et al*. 2007b; 2013b).

The BNI-capacity of root systems arises from their ability to release two categories of BNIs – a. hydrophobic-BNIs and b. hydrophilic-BNIs. These two BNI fractions differ in their mobility in the soil and their solubility in water; the hydrophobic-BNIs may remain close to the root as they could be strongly adsorbed on the soil particles, increasing their persistence. The mobility of the hydrophobic-BNIs is *via* diffusion across a concentration gradient; and thus is likely to be confined to the rhizosphere (Raynaud 2010; Subbarao *et al*. 2013a). In contrast, the hydrophilic-BNIs may move further from the point of release due to their solubility in water, and this may improve their capacity to control nitrification beyond the rhizosphere (Subbarao *et al*. 2013a). The relative contribution of hydrophobic-BNIs *vs*. hydrophilic-BNIs to the BNI-capacity may differ among plant species. For *Brachiaria* grasses, both fractions make equal contribution to the BNI-capacity; for sorghum, the hydrophobic-BNIs

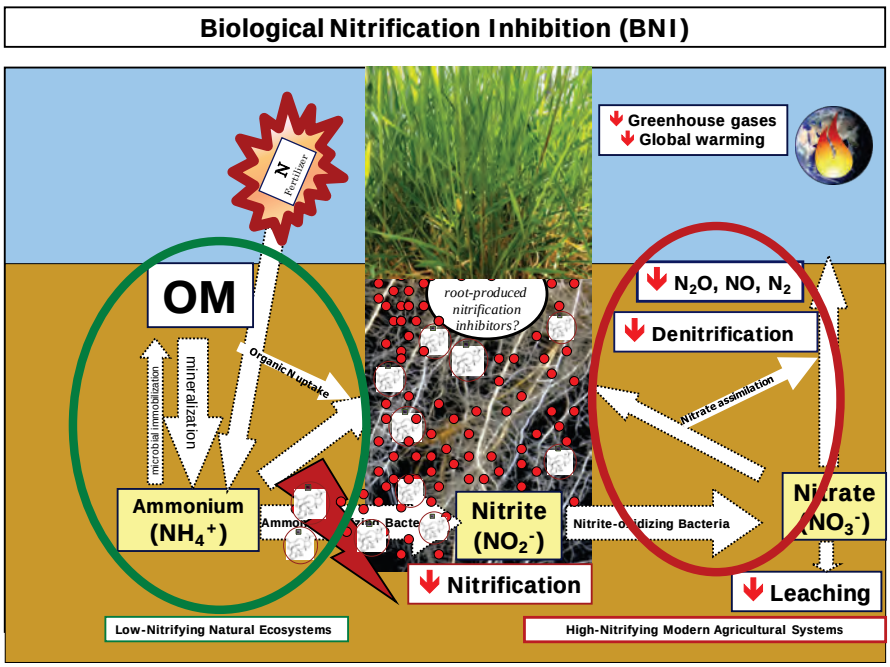

Figure 1. Schematic representation of the biological nitrification inhibition (BNI) interfaces with the N cycle. The BNI exuded by roots inhibits nitrification that converts NH_4+ to NO_2^-. In ecosystems with large amounts of BNI (*e.g.* brachialactone) such as *Brachiaria* grasses, the flow of N from NH4+ to NO_3^- is restricted, and it is NH_4^+ and microbial N rather than NO_3^- that accumulates in the soil. In systems with little or no BNI, such as modern agricultural systems, nitrification occurs rapidly, leaving little time for plant roots to absorb NO_3^-, thus NO_3^- lost from the system through denitrification and leaching (adapted from Subbarao *et al.*, 2012).

play a dominant role in determining the BNI-capacity, whereas in wheat, hydrophilic-BNIs determine the root-system's inhibitory capacity (GV Subbarao and T Tsehaye, unpublished).

For *Brachiaria* spp., the amount of inhibitors released from root systems could be substantial. Based on the BNI-activity release rates observed (17 to 50 ATU/g root dry wt./d) and assuming the average live root biomass from a long-term grass pasture at 1.5 Mg/ha (Rao 1998), it was estimated that BNI-activity of 2.6 x 10^6 to 7.5 x 10^6 ATU /ha/d is potentially released (Subbarao *et al.* 2009a); this amounts to an inhibitory potential equivalent to that is achieved by the application of 6.2 to 18.0 kg of nitrapyrin/ha/yr, which is large enough to have a significant influence on the function of nitrifier population and nitrification rates in the soil (Subbarao *et al*. 2009a). Field studies indicate a 90% decline in soil ammonium oxidation rates due to extremely small populations of nitrifiers (ammonia oxidizing bacteria and ammonia oxidizing archaea) within 3 years of establishment of *B. humidicola* (Fig. 3). Nitrous oxide emission was suppressed by >90% in field plots of *B. humidicola* compared to soybean, which lacks BNI-capacity in its root systems (Subbarao *et al*. 2009a).

Chemical identities of BNIs and their mode of inhibitory action

The major nitrification inhibitor released from the roots of *B. humidicola* is a cyclic diterpene, named 'brachialactone' (Subbarao *et al*. 2009a). This compound has a dicyclopenta [a,d] cyclooctane skeleton (5-8-5 ring system) with a γ-lactone ring bridging one of the five-membered rings and the eight-membered ring (Fig. 4) (Subbarao *et al*. 2009a). Brachialactone with an IC_{80} (concentration for 80% inhibition in the bioassay) of 10.6 μM, is considered to be a potent nitrification inhibitor when compared with nitrapyrin (IC_{80}: 5.8 μM) or dicyandiamide (DCD, IC_{80}:2200 μM), two of the synthetic nitrification inhibitors most commonly used in production agriculture. Brachialactone inhibits *Nitrosomonas* spp. by blocking ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), but appears to have a relatively stronger effect on the AMO than on the HAO enzymatic pathway. About 60 to 90% of the inhibitory activity released from the roots of *B. humidicola* is due to brachialactone. Release of brachialactone is a regulated plant function, triggered and sustained by the availability of NH_4^+ in the root environment (Subbarao *et al*. 2007a; 2009a). Also, brachialactone release is restricted to those roots that are directly exposed

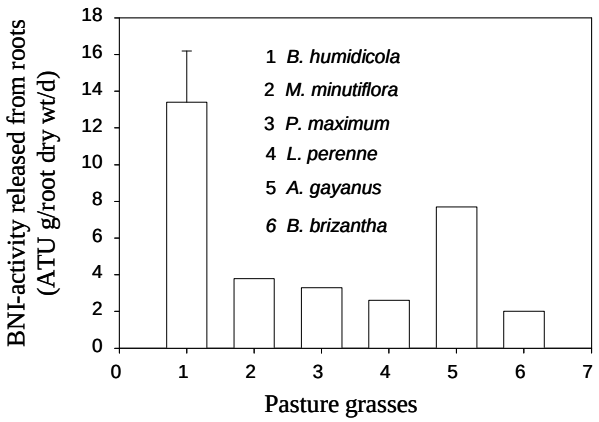

Figure 2. BNI activity released from intact roots of various pasture grasses grown in sand-vermiculite (3:1 v/v) culture for 60 days (based on Subbarao *et al.* 2007b). Vertical bar represents LSD (0.05).

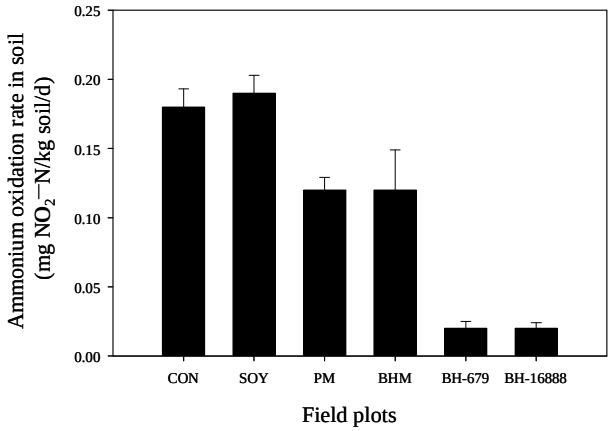

Figure 3. Soil ammonium oxidation rates (mg of NO_2^-/kg soil/d) in field plots planted with tropical pasture grasses (differing in BNI capacity) and soybean (lacking BNI capacity in roots) [covering 3 years from establishment of pasture (September 2004-November 2007)]; for soybean, two planting seasons every year and after six seasons of cultivation: CON, control (plant-free) plots; SOY, soybean; PM, *P. maximum*; BHM, *Brachiaria* hybrid 'Mulato'; BH-679, *B. humidicola* CIAT 679 (standard); BH-16888, *B. humidicola* accession CIAT 16888 (a germplasm accession). "BHM" is an apomictic hybrid that contains germplasm from *B. ruziziensis*, *B. decumbens*, and *B. brizantha*, and that it does NOT contain any contribution from *B. humidicola*. Values are means +/- s.e. of three replications (adapted from Subbarao *et al.* 2009a).

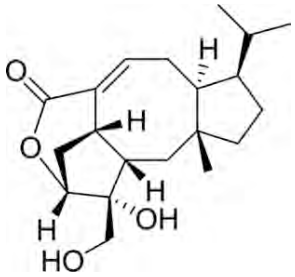

Figure 4. Chemical structure of brachialatone, the major nitrification inhibitors isolated from root exudates of *B. humidicola* (from Subbarao *et al.* 2009a).

to NH_4^+, and not in the entire root system, suggesting a localized release response (Subbarao *et al*. 2009a).

Genetic improvement of BNI-capacity of pasture grasses

Significant genetic variability (ranging from 7.1 to 46.3 ATU/g root dry wt./d) exists for the BNI-capacity in *B. humidicola*, indicating potential for genetic manipulation of the BNI-capacity by conventional plant breeding (Subbarao *et al*. 2007b; 2009b). Recent findings suggest substantial genetic variability for brachialactone release among *B. humidicola* germplasm accessions, nearly 10-fold differ-ences, suggesting the potential for breeding high brachialactone-capacity genotypes in *Brachiaria*. Efforts are underway to develop molecular markers for brachial-actone release capacity in *Brachiaria* spp.

Conclusions

Sustainable intensification of grasslands and feed-crop production systems is needed to meet the global demands for meat and milk, particularly in developing countries. As the demand for meat and milk are expected to double by 2050 (Herrero *et al*. 2009), there will be further efforts to intensify grasslands and feed-crop based systems. Most of these increases in productivity are however achieved through massive inputs of N-fertilizer. Nearly 70% of the 150 Tg N applied to global agricultural systems is lost, largely due to the high-nitrifying nature of soil environments (Tilman *et al*. 2001; Subbarao *et al*. 2013b). As nitrification and denitrification are the primary biological drivers of NO_3^-, N_2O and NO production (*i.e.* reactive N forms largely responsible for environmental pollution), suppressing nitrification has the potential to reduce N losses and to retain soil-N for longer periods in the grassland systems. The BNI function in some forage grasses and feed-crops such as sorghum can be exploited using both genetic and crop and/or production system-based management to design low-nitrifying agronomic environments to improve NUE. Also, the high BNI-capacity in the forage grass(es) *Brachiaria* spp. can be exploited for the benefit of feed-crop systems such as maize that receive most of the N-fertilization but do not have an intrinsic BNI-capacity in their root systems. This may be achieved by integrating *Brachiaria* pastures of high-BNI capacity with maize production using agro-pastoral systems (Subbarao *et al*. 2013b). In grazed grassland systems, most of the plant-protein-N is excreted by the livestock (through urine) and thus returned to the soil. Grassland systems that retain the N excreted by the livestock would be better able to maintain and sustain their productivity over time. Grazing animals usually deposit urine and dung in a random, patchy manner which makes control of nitrification using synthetic nitrification inhibitors potentially difficult. The inducible BNI function in forage grasses could be a more effective way to control nitrification, to sustain system productivity and to minimize environmental degradation under these circumstances. It appears likely that the control of nitrification by using BNI in grassland systems could be enhanced by conventional plant breeding or potentially by genetic engineering. Many forage-grasses develop extensive root systems and are perennial (Rao *et al*. 2011); if this is combined with high-BNI capacity, grassland systems would suppress nitrifier activity in the soil and retain N for more effective use by grasses by reducing N loss.

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